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Overexpression of epidermal growth factor receptor (EGFR), HER-2/*neu*, and *myc* oncogenes are some of the well described patterns of genetic changes that occur frequently in breast cancer. In addition, deletions of chromosomal loci that are thought to be associated with putative tumor suppressors and other genes, also contribute to more aggressive phenotype and metastasis of breast cancer. These genetic changes have important prognostic implication in the clinical outcome of breast cancer. Our laboratory is interested in exploring the role of *PML*, a novel tumor suppressor, on the regulation of EGFR expression in breast cancer. We show recently that *PML* suppresses the clonogenicity and tumorigenicity of cells derived from acute promyelocytic leukemia (APL). Furthermore, *PML* suppresses the transformation of REF and NIH3T3 cells by oncogenes. We also show that *PML* specifically represses the activity of EGFR gene promoter. These results suggest that *PML* is a tumor suppressor gene and that the chromosomal translocation in APL involving *PML* and retinoic acid receptor-α (RARα) disrupts the biological function of *PML* as a tumor suppressor. In the current study, we propose to investigate the interaction between *PML* and EGFR, and its consequence in the development of breast cancer.

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Introduction

Breast cancer is one of the most prevalent malignancies in women and accounts for the highest morbidity among women suffering from cancers (1). The oncogenic development of breast cancer is accompanied by genetic alterations of multiple oncogenes, tumor suppressor genes, and other factors. The collaborative effects of these transforming proteins induce alterations in the cellular biochemical, physiological, and genetic processes, which include both gene induction and gene repression, alterations in growth requirement, and acquisition of metastatic potential. These changes may lead to neoplastic transformation of the mammary tissue. The complexity and heterogeneity of the array of genetic, hormonal, and dietary factors that may contribute to the etiology of breast cancer is further confounded by the lack of information on specific genetic mutations associated with the initiation and progression of the disease.

Overexpression of the epidermal growth factor receptor (EGFR), HER-2/*neu*, and *myc* oncogenes are some of the well described genetic changes that frequently occur in breast cancer (2-4). In addition, deletions of chromosomal loci that are thought to be associated with putative tumor suppressors including the p53, BRCA1 and BRCA2 genes, also contribute to a more aggressive phenotype of breast cancer (5). These genetic changes have important prognostic implication in the clinical outcome of breast cancer.

Epidermal growth factor (EGF) is a small polypeptide that stimulates cell proliferation in both cell culture and in intact animals (6). EGF has been shown to promote both normal and neoplastic growth of mammary tissue in rodents (7) and in human breast cancer cells in culture (8-10). The biological effects of EGF are mediated through high affinity binding to EGFR, which is a 170 kDa membrane receptor tyrosine kinase (6). There is great interest in the study of EGFR in human breast cancer, however, the clinical relationships and prognostic value of the receptor in breast cancer are still unclear (11).

The transforming growth factor-α (TGFα), which bears considerable sequence homology to EGF and is produced by many transformed cells, also binds to EGFR and mimics the action of EGF (6,12). Expression of EGFR in breast cancer cells is regulated by mitogenic growth factors, and also by the superfamily of nuclear hormone receptors, which includes estrogen, progesterone, glucocorticoids, and retinoic acid receptors (13). Therefore, optimal regulation of EGFR expression is a complex process involving the coordinate interaction of several heterologous growth factors and hormones, whereby the proliferation of normal and neoplastic breast cells can be modulated. Although the clinical significance and prognostic value of EGFR in human breast cancer are unresolved, the involvement of EGFR in the growth of normal and malignant human mammary cells indicates that it may play a critical role in the oncogenesis of human breast carcinomas. Further evidence of the importance of EGFR in the development of breast cancer has been shown in transgenic mice studies where the overexpression of TGFα causes a significant increase in the occurrence of mammary carcinomas (14-16).

Specific chromosomal abnormalities occur frequently in acute and chronic leukemias (17). These cytogenetic aberrations are thought to contribute to leukemogenesis. Furthermore, significant differences in the type of genes involved in chromosomal translocations in acute leukemias and chronic leukemias have also been observed. For example, study of the recombination of *bcr* and *abl* genes in the t(9;22) of CML, and the *myc* and immunoglobulin genes in the t(8;14) of Burkitt lymphoma, has led to the identification of new fusion genes involved in the neoplastic transformation of these hematopoietic tumors (17-19). It has also been shown that a chromosomal translocation breakpoint t(15;17) occurs in over 90% of all patients with acute promyelocytic leukemia (APL), a subtype of acute myeloblastic leukemia (20-23). The recombination involves the *PML* (*myl*) gene on chromosome 15 and the retinoic acid receptor-α (RARα) on chromosome 17. The chimera *PML*/RARα and RARα/*PML* genes are formed as a result of the reciprocal translocation between the *PML* and RARα loci (20-22, 24). The

PML/RARα cDNA has been isolated and shown to encode a fusion protein that is retinoic acid responsive and exhibits transactivation potential in a cell type- and promoter-specific manner differing from the wild-type RARα (25-27). Since the administration of all-trans retinoic acid to APL patients leads to rapid achievement of remission, it has been suggested that disruption of RARα may be part of the underlying pathogenesis of APL (28-30). The biological function and etiologic implications for the *PML* gene is not known and leaves open the question of its role in APL. Characterization of *PML* reveals that it is a putative zinc finger protein and transcription factor that shares homology with a newly recognized family of proteins that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1) (25-27,31). Expression of *PML* is found in a variety of fetal and adult tissues including brain, gut, liver, lung, muscle, placenta, and testes (31,32).

Our laboratory, in collaboration with Dr. Kun-Sang Chang at the University of Texas, M.D. Anderson Cancer Center, investigated the role of t(15;17) chromosomal translocation in the leukemogenesis and the emergence of multidrug resistance in acute promyelocytic leukemia (APL). We demonstrated that *PML* suppresses the clonogenicity and tumorigenicity of the APL-derived NB4 cells in soft agar (33). Cells transfected with expression vector containing *PML* showed more than 50% reduction in colonies formed on soft agar. Cells transfected with control plasmid (pSG5) and *PML* mutants (PSG5*PML*mut and pSG5*PML*/RARα) did not show inhibition of colony growth. Furthermore, we also show that *PML* suppresses the transformation of REF and NIH3T3 cells by oncogenes. In all of these experiments the fusion product *PML*/RARα fails to suppress the tumorigenic growth of NB4 cells as well as the transformation of the REF and NIH3T3 cells. These results suggest that the translocation of APL inactivated the biological function of *PML* as a tumor suppressor and that this molecular alteration may be a precipitating event in the development of APL.

To assess the putative function of *PML* and *PML*/RARα as a transcription factor, we examined their ability to transactivate promoter activity. Our results showed that *PML* significantly represses the activity of the EGFR gene promoter. However, cotransfection with *PML*/RARα exhibited significantly decreased suppression of the EGFR promoter. These studies showed that *PML* acts to suppress the transcriptional activity of specific gene promoter and that mutant *PML* (*PML*/RARα) lost its transrepression function.

The results of our experiments suggest that the negative regulation of EGFR expression by *PML* may attenuate growth of mammary tissue normally, while genetic alterations of PML may lead to the neoplastic growth of the tissue. Our laboratory, therefore is interested in exploring the role of *PML*, a novel growth suppressor, and its regulation of EGFR expression in the genetic and molecular etiology of breast cancer.

Body

Since I received the Career Development Award from the USAMRDC, which supports my salary, I have focused on the analysis of the effects of *PML* on the EGFR promoter. I have performed deletion analysis and attempted to identify the core promoter sequence element that is required for *PML*-mediated repression of the EGFR promoter.

To identify the potential regions required for *PML* mediated regulation of the EGFR gene expression, a series of deletion fragments of the EGFR reporter gene promoter ligated 5' to the bacterial chloramphenicol acetyltransferase (CAT) gene were obtained from Dr. Alfred Johnson (National Cancer Institute, Bethesda, MD), and tested for promoter activity by cotransfection with an expression vector for *PML* into the human adrenal SW13 cells. The structure of the various deletions are shown in Fig. 1 (34). The results of this analysis in SW13 cells show that the basal EGFR promoter activity is reduced by approximately 80% with the deletion of the region between

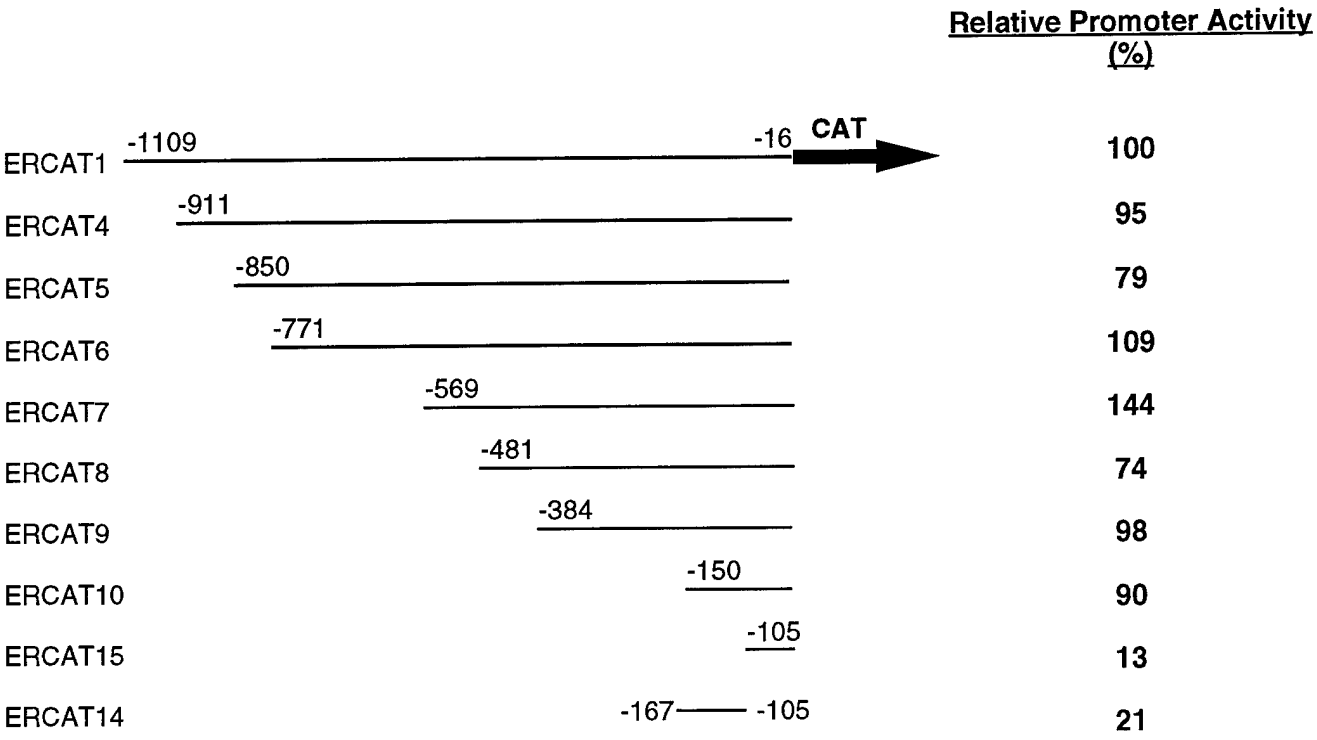

Fig.1. Deletion mutants of the EGFR receptor gene promoter. The series of chimeric plasmids were constructed and transfected into the human adrenal SW13 cells. Cells were harvested for CAT analysis approximately 36 to 48 hr after transfection. Promoter activity was determined and is expressed relative to ERCAT1 which contains a 1.1 kb promoter DNA fragment.

-150 and -105 (Fig. 1). Promoter activity is also substantially reduced with the removal of the region between -105 and -16. Deletion of the sequences from -150 to -105 and the region close to the transcription start site from -150 to -16 affected the promoter function, suggesting that these regions may be important for the basal activity of the EGFR promoter.

To characterize the region or specific sequences that may be required for regulation by *PML*, the above deletion constructs were cotransfected with expression vectors containing either the full length cDNA for *PML* or the mutant *PML*/RARα fusion, driven by the SV40 early gene promoter in to SW13 cells. Consistent with results previous studies (33), we observed that *PML* suppresses the EGFR promoter activity (Fig. 2). Surprisingly, *PML*-mediated suppression occurs in all the constructs, including those that possessed low level basal promoter activities (ERCAT 14 and 15). However, loss of suppression or stimulation of the promoter was observed in the presence of the mutant *PML*/RARα fusion construct. When the region between -167 and -16 was deleted, no effect of *PML*/RARα on the promoter was observed. These results suggest that *PML*-mediated regulation of the EGFR gene may not involve direct binding of *PML* to the promoter, since extensive deletion of the promoter did not affect the suppression by *PML*. Cotrnsfection with the mutant *PML*/RARα fusion revealed that it failed to stimulate the EGFR promoter when the region between -167 and -16 was deleted, indicating that this core region may be important for regulation by *PML*. Since this region is also needed for basal promoter activity and no apparent consensus sequence was required for the effects of *PML*, therefore, *PML* may interact directly with the general transcription factors to repress the activity of the promoter.

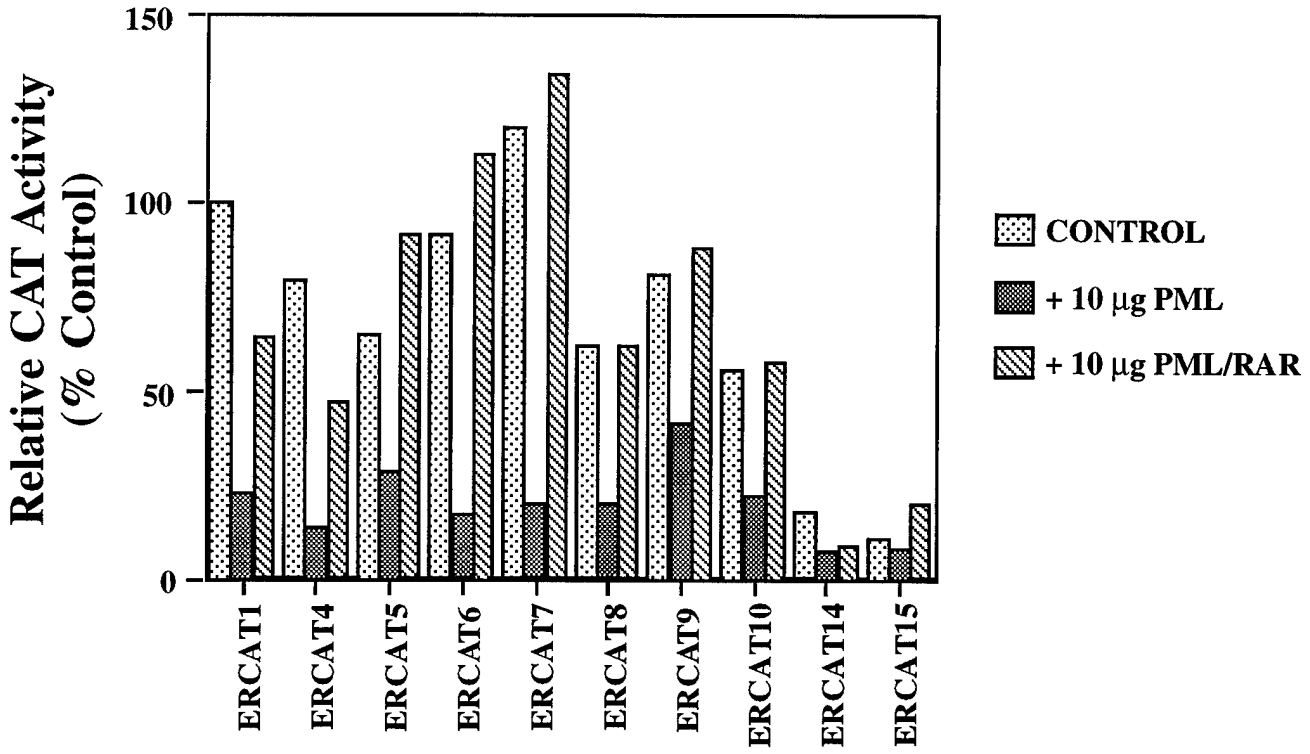

Fig. 2. Effects of *PML* and *PML*/RARα on deletion mutants of the EGFR gene promoter-CAT chimeras. Expression vectors containing cDNAs for *PML* or *PML*/RARα were cotransfected with the various deletion mutants of the EGFR gene promoter-CAT chimeras into SW13 cells. Cells were harvested for CAT assay approximately 36 to 48 hr after transfection. Promoter activity was determined and the effects of *PML* or *PML*/RARα were expressed relative to ERCAT1 which contains a 1.1 kb promoter DNA fragment.

Conclusions

We have shown by deletion analysis that the region of the EGFR gene promoter required for *PML*-mediated suppression overlaps with the basal promoter sequence between -167 and -16 (relative to the transcription start site), and suggest that *PML* may repress the basal promoter complex. It is conceivable that *PML* may interact with components of the basal or general transcription factors and act as a general transcription repressor that regulates the expression of specific genes possessing high GC nucleotides content in their promoters, like that of the EGFR gene.

However, our results with the mutant *PML*/RARα fusion construct are not unambiguous, in that we cannot rule out whether the RARα portion of the fusion construct have a direct effect on the promoter. We are trying to make a nonfunctional deletion mutant of *PML* for future studies to ascertain the effects of *PML* on the EGFR promoter. In addition, we will also assess independently the effects of RARα on the EGFR gene promoter to determine whether RARα may influence the promoter activity.

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